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(71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants (for US only) : NORRIS, Fanny [DK/DK]; NORRIS, Kjeld [DK/DK]; Ahlmanns Allé 34, DK-2900 Hellerup (DK). BJØRN, Søren, Erik [DK/DK]; Marie Grubbes Allé 47, DK-2800 Lyngby (DK). PETERSEN, Lars, Christian [DK/DK]; Havevej 4, DK-2970 Hørsholm (DK). OLSEN, Ole, Hvilsted [DK/DK]; Bækkeskovvej 38, DK-2700 Brønshøj (DK).

(74) Common Representative: NOVO NORDISK A/S; Novo Allé, DK-2880 Bagsværd (DK).

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(54) Title: A HUMAN KUNITZ-TYPE PROTEASE INHIBITOR VARIANT

X^1 Ser Trp Cys Leu Thr Pro Ala Asp X^2 Gly X^3 Cys X^4 X^5 X^6 X^7 X^8 X^9 Phe Tyr Tyr Asn Ser Val Ile Gly Lys Cys Arg Pro Phe X^{10} Tyr X^{11} Gly Cys X^{12} X^{13} X^{14} Glu Asn Asn Phe X^{15} Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys Lys X^{16}

(I)

Abstract

A variant of human Kunitz-type protease inhibitor domain III of tissue factor protease inhibitor (TFPI), the variant comprising the amino acid sequence (I) wherein X^1 represents H or 1-5 naturally occurring amino acid residues except Cys, X^2-X^{15} each independently represents a naturally occurring amino acid residue, and X^{16} represents OH or 1-5 naturally occurring amino acid residues except Cys, with the proviso that at least one of the amino acid residues X^1-X^{16} is different from the corresponding amino acid residue of the native sequence

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A HUMAN KUNITZ-TYPE PROTEASE INHIBITOR VARIANT

FIELD OF INVENTION

The present invention relates to a variant of a human Kunitz-type protease inhibitor domain, DNA encoding the variant, a method of producing the variant and a pharmaceutical composition containing the variant.

BACKGROUND OF THE INVENTION

Polymorphonuclear leukocytes (neutrophils or PMNs) and mononuclear phagocytes (monocytes) play an important part in tissue injury, infection, acute and chronic inflammation and wound healing. The cells migrate from the blood to the site of inflammation and, following appropriate stimulation, they release oxidant compounds ($O_2^{\bullet}$, O_2^-, H_2O_2 and HOCl) as well as granules containing a variety of proteolytic enzymes. The secretory granules contain, i.a., alkaline phosphatase, metalloproteinases such as gelatinase and collagenase and serine proteases such as neutrophil elastase, cathepsin G and proteinase 3.

Latent metalloproteinases are released together with tissue inhibitor of metalloproteinase (TIMP). The activation mechanism has not been fully elucidated, but it is likely that oxidation of thiol groups and/or proteolysis play a part in the process. Also, free metalloproteinase activity is dependent on inactivation of TIMP.

In the azurophil granules of the leukocytes, the serine proteases neutrophil elastase, cathepsin G and proteinase-3 are packed as active enzymes complexed with glucosaminoglycans. These [illegible] release the active enzymes [illegible] the protease [illegible] large amounts of the inhibitors α_1-proteinase inhibitor (α_1-PI) and α_1-chymotrypsin inhibitor (α_1-ChI) are found in plasma. However, the PMNs are able to inactivate the inhibitors locally.

Thus, α_1-PI which is the most important inhibitor of neutrophil elastase is sensitive to oxidation at the reactive centre (Met-358) by oxygen metabolites produced by triggered PMNs. This reduces the affinity of α_1-PI for neutrophil elastase by approximately 2000 times.

After local neutralisation of α_1-PI, the elastase is able to degrade a number of inhibitors of other proteolytic enzymes. Elastase cleaves α_1-ChI and thereby promotes cathepsin G activity. It also cleaves TIMP, resulting in tissue degradation by metalloproteinases. Furthermore, elastase cleaves antithrombin III and heparin cofactor II, and tissue factor pathway inhibitor (TFPI) which probably promotes clot formation. On the other hand, the ability of neutrophil elastase to degrade coagulation factors is assumed to have the opposite effect so that the total effect of elastase is unclear. The effect of neutrophil elastase on fibrinolysis is less ambiguous. Fibrinolytic activity increases when the elastase cleaves the plasminogen activator inhibitor and the α_2 plasmin inhibitor. Besides, both of these inhibitors are oxidated and inactivated by O_2 metabolites.

PMNs contain large quantities of serine proteases, and about 200 mg of each of the leukocyte proteases are released daily to deal with invasive agents in the body. Acute inflammation leads to a many-fold increase in the amount of enzyme released. Under normal conditions, proteolysis is kept at an acceptably low level by large amounts of the inhibitors α_1-PI, α_1-ChI and α_2 macroglobulin. There is some indication, however, that a number of chronic diseases is caused by pathological proteolysis due to overstimulation of the PMNs, for instance caused by autoimmune response, chronic infection, tobacco smoke or other irritants, etc.

Aprotinin (bovine pancreatic trypsin inhibitor) is known to inhibit various serine proteases, including trypsin, chymotrypsin, plasmin and kallikrein, and is used

therapeutically in the treatment of acute pancreatitis, various states of shock syndrome, hyperfibrinolytic haemorrhage and myocardial infarction (cf., for instance, J.E. Trapnell et al, Brit. J. Surg. 61, 1974, p. 177; J. McMichan et al., Circulatory shock 9, 1982, p. 107; L.M. Auer et al., Acta Neurochir. 49, 1979, p. 207; G. Sher, Am. J. Obstet. Gynecol. 129, 1977, p. 164; and B. Schneider, Artzneim.-Forsch. 26, 1976, p. 1606). Administration of aprotinin in high doses significantly reduces blood loss in connection with cardiac surgery, including cardiopulmonary bypass operations (cf., for instance, B.P. Bidstrup et al., J. Thorac. Cardiovasc. Surg. 97, 1989, pp. 364-372; W. van Oeveren et al., Ann. Thorac. Surg. 44, 1987, pp. 640-645). It has previously been demonstrated (cf. H.R. Wenzel and H. Tschesche, Angew. Chem. Internat. Ed. 20, 1981, p. 295) that certain aprotinin analogues, e.g. aprotinin(1-58, Val15) exhibits a relatively high selectivity for granulocyte elastase and an inhibitory effect on collagenase, aprotinin (1-58, Ala15) has a weak effect on elastase, while aprotinin (3-58, Arg15, Ala17, Ser42) exhibits an excellent plasma kallikrein inhibitory effect (cf. WO 89/10374).

However, when administered in vivo, aprotinin has been found to have a nephrotoxic effect in rats, rabbits and dogs after repeated injections of relatively high doses of aprotinin (Bayer, Trasylol, Inhibitor of proteinase; E. Glaser et al. in "Verhandlungen der Deutschen Gesellschaft für Innere Medizin, 78. Kongress", Bergmann, München, 1972, pp. 1612-1614). The nephrotoxicity (i.a. appearing in the form of lesions) observed for aprotinin might be ascribed to the accumulation of aprotinin in the proximal tubulus cells of the kidneys as a result of the high positive net charge of aprotinin which causes it to be bound to the negatively charged surfaces of the tubuli.. This nephrotoxicity makes aprotinin less suitable for clinical purposes, in particular [illegible] (see [illegible] inhibitor such [illegible] cardiopulmonary [illegible] operations). Besides, aprotinin is a bovine protein which may therefore contain one or more epitopes which may give rise to an

undesirable immune response on administration of aprotinin to humans.

It is therefore an object of the present invention to identify human protease inhibitors of the same type as aprotinin (i.e. Kunitz-type inhibitors) with a similar inhibitor profile or modified to exhibit a desired inhibitor profile.

SUMMARY OF THE INVENTION

The present invention relates to a variant of human Kunitz-type protease inhibitor domain III of tissue factor pathway inhibitor (TFPI), the variant comprising the following amino acid sequence

X^1 Ser Trp Cys Leu Thr Pro Ala Asp X^2 Gly X^3 Cys X^4 X^5 X^6 X^7 X^8 X^9 Phe Tyr Tyr Asn Ser Val Ile Gly Lys Cys Arg Pro Phe X^{10} Tyr X^{11} Gly Cys X^{12} X^{13} X^{14} Glu Asn Asn Phe X^{15} Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys Lys X^{16} (SEQ ID No. 1)

wherein X^1 represents H or 1-5 naturally occurring amino acid residues except Cys, X^2-X^{15} each independently represents a naturally occurring amino acid residue except Cys, and X^{16} represents OH or 1-5 naturally occurring amino acid residues except Cys, with the proviso that at least one of the amino acid residues X^1-X^{16} is different from the corresponding amino acid residue of the native sequence.

In the present context, the term "naturally occurring amino acid residue" is intended to indicate any one of the 20 commonly occurring amino acids, i.e. Ala, Val, Leu, Ile Pro, Phe, Trp, Met, Gly, Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg and His.

TFPI, also known as extrinsic pathway inhibitor (EPI) or lipoprotein associated coagulation inhibitor (LACI), has been isolated by Broze et al. (Proc. Natl. Acad. Sci. USA 84, 1987, pp. 1886-1890 and EP 300 988) and the gene coding for the

protein has been cloned, cf. EP 318 451. Analysis of the secondary structure of the protein has shown that the protein has three Kunitz-type inhibitor domains, from amino acid 22 to amino acid 79 (I), from amino acid 93 to amino acid 150 (II) and from amino acid 185 to amino acid 242 (III). Kunitz-type domain I of TFPI has been shown to bind TF/FVIIa, while Kunitz-type domain II has been shown to bind to FXa (Girard et al., Nature 338, 1989, pp. 518-520).

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor profile of TFPI Kunitz-type domain III so that it preferentially inhibits neutrophil elastase, cathepsin G and/or proteinase-3. Furthermore, it may be possible to construct variants which specifically inhibit enzymes involved in coagulation or fibrinolysis (e.g. plasmin or plasma kallikrein) or the complement cascade.

One advantage of TFPI Kunitz-type domain III is that it has a negative net charge as opposed to aprotinin which, as indicated above, has a strongly positive net charge. It is therefore possible to construct variants of the invention with a lower positive net charge than aprotinin, thereby reducing the risk of kidney damage on administration of large doses of the variants. Another advantage is that, contrary to aprotinin, it is a human protein (fragment) so that undesired immunological reactions on administration to humans are significantly reduced.

DETAILED DISCLOSURE OF THE INVENTION

Examples of preferred variants of Kunitz-type domain III of TFPI are variants wherein X^1 is Gly-Pro; or wherein X^2 is an amino acid residue selected from the group consisting of Ala, Arg,

wherein [illegible] wherein [illegible] amino acid residue selected from the group consisting of Pro, Thr, Leu, Arg, Val and Ile, in particular wherein X^3 is Pro or Leu; or wherein X^4 is

an amino acid residue selected from the group consisting of Lys, Arg, Val, Thr, Ile, Leu, Phe, Gly, Ser, Met, Trp, Tyr, Gln, Asn and Ala, in particular wherein X^4 is Lys, Val, Leu, Ile, Thr, Met, Gln or Arg; or wherein X^5 is an amino acid residue selected from the group consisting of Ala, Gly, Thr, Arg, Phe, Gln and Asp, in particular wherein X^5 is Ala, Thr, Asp or Gly; or wherein X^6 is an amino acid residue selected from the group consisting of Arg, Ala, Lys, Leu, Gly, His, Ser, Asp, Gln, Glu, Val, Thr, Tyr, Phe, Asn, Ile and Met, in particular wherein X^6 is Arg, Phe, Ala, Asn, Leu or Tyr; or wherein X^7 is an amino acid residue selected from the group consisting of Ile, Met, Gln, Glu, Thr, Leu, Val and Phe, in particular wherein X^7 is Ile or Glu; or wherein X^8 is an amino acid residue selected from the group consisting of Ile, Thr, Leu, Asn, Lys, Ser, Gln, Glu, Arg, Pro and Phe, in particular wherein X^8 is Ile or Asn; or wherein X^9 is an amino acid residue selected from the group consisting of Arg, Ser, Ala, Gln, Lys and Leu, in particular wherein X^9 is Arg; or wherein X^{10} is an amino acid residue selected from the group consisting of Gln, Pro, Phe, Ile Lys, Trp, Ala, Thr, Leu, Ser, Tyr, His, Asp, Met, Arg and Val, in particular wherein X^{10} is Val or Lys; or wherein X^{11} is Ser or Gly; or wherein X^{12} is an amino acid residue selected from the group consisting of Gly, Met, Gln, Glu, Leu, Arg, Lys, Pro and Asn, in particular wherein X^{12} is Arg or Glu; or wherein X^{13} is Ala or Gly; or wherein X^{14} is an amino acid residue selected from the group consisting of Lys, Asn and Asp, in particular wherein X^{14} is Lys or Asn; or wherein X^{15} is an amino acid residue selected from the group consisting of Val, Tyr, Asp, Glu, Thr, Gly, Leu, Ser, Ile, Gln, His, Asn, Pro, Phe, Met, Ala, Arg, Trp and Lys, in particular wherein X^{15} is Lys or Thr; or wherein X^{16} is Gly. In a preferred embodiment, X^1 is Lys-Pro and X^{16} is Gly, while X^2-X^{15} are as defined above.

Variants of TFPI Kunitz-type domain III of the invention should preferably not contain a Met residue in the protease binding region (i.e. the amino acid residues represented by X^3-X^{14}). By analogy to α1-PI described above, a Met residue in any one of these positions would make the inhibitor sensitive to oxidative

inactivation by oxygen metabolites produced by PMNs, and conversely, lack of a Met residue in these positions should render the inhibitor more stable in the presence of such oxygen metabolites.

A currently preferred variant of the invention is one in which one or more of the amino acid residues located at the protease-binding site of the Kunitz domain (i.e. one or more of X^3-X^{14} corresponding to positions 13, 15, 16, 17, 18, 19, 20, 34, 39, 40, 41 and 46 of aprotinin) are substituted to the amino acids present in the same positions of native aprotinin. This variant comprises the following amino acid sequence

Gly Pro Ser Trp Cys Leu Thr Pro Ala Asp Arg Gly Pro Cys Lys Ala Arg Ile Ile Arg Phe Tyr Tyr Asn Ser Val Ile Gly Lys Cys Arg Pro Phe Val Tyr Gly Gly Cys Arg Ala Lys Glu Asn Asn Phe Lys Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys Lys Gly (SEQ ID No. 2).

In another aspect, the invention relates to a DNA construct encoding a human Kunitz-type inhibitor domain variant according to the invention. The DNA construct of the invention may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

Alternatively, it is possible to use genomic or cDNA coding for TFPI Kunitz-type domain III (e.g. obtained by screening a genomic or cDNA library for DNA coding for TFPI using synthetic

[illegible] more sites corresponding to the site(s) at which it is desired to introduce amino acid substitutions, e.g. by site-directed

mutagenesis using synthetic oligonucleotides encoding the desired amino acid sequence for homologous recombination in accordance with well-known procedures.

In a still further aspect, the invention relates to a recombinant expression vector which comprises a DNA construct of the invention. The recombinant expression vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the TFPI Kunitz-type domain III variant of the invention should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the TFPI Kunitz-type domain III variant of the invention in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814) or the adenovirus 2 major late promoter. Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4, 599, 311) or ADH2-4c (Russell et al., Nature 304,

1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the tpiA promoter.

The DNA sequence encoding the TFPI Kunitz-type domain III variant of the invention may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant expression vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An examples of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication, or (when the host cell is a yeast cell) the yeast plasmid 2μ replication genes REP 1-3 and origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hygromycin or methotrexate, or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130.

The procedures used to ligate the DNA sequences coding for the TFPI Kunitz-type domain III variant of the invention, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication,

[illegible] instance improved [illegible] manual, Cold Spring Harbor, New York, 1989).

The host cell into which the expression vector of the invention is introduced may be any cell which is capable of producing the TFPI Kunitz-type domain III variant of the invention and is preferably a eukaryotic cell, such as a mammalian, yeast or fungal cell.

The yeast organism used as the host cell according to the invention may be any yeast organism which, on cultivation, produces large quantities of the TFPI Kunitz-type domain III variant of the invention. Examples of suitable yeast organisms are strains of the yeast species Saccharomyces cerevisiae, Saccharomyces kluyveri, Schizosaccharomyces pombe or Saccharomyces uvarum. The transformation of yeast cells may for instance be effected by protoplast formation followed by transformation in a manner known per se.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159, 1982, pp. 601-621; Southern and Berg, J. Mol. Appl. Genet. 1, 1982, pp. 327-341; Loyter et al., Proc. Natl. Acad. Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and Pearson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Neumann et al., EMBO J. 1, 1982, pp. 841-845.

Alternatively, fungal cells may be used as host cells of the invention. Examples of suitable fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus spp. for the expression of proteins is described in, e.g., EP 238 023.

The present invention further relates to a method of producing a TFPI Kunitz-type domain III variant according to the

invention, the method comprising culturing a cell as described above under conditions conducive to the expression of the variant and recovering the resulting variant from the culture.

The medium used to cultivate the cells may be any conventional medium suitable for growing mammalian cells or fungal (including yeast) cells, depending on the choice of host cell. The variant will be secreted by the host cells to the growth medium and may be recovered therefrom by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulfate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography or affinity chromatography, or the like.

The present invention also relates to a pharmaceutical composition comprising a TFPI Kunitz-type domain III variant of the invention together with a pharmaceutically acceptable carrier or excipient. In the composition of the invention, the variant may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. The composition may typically be in a form suited for systemic injection or infusion and may, as such, be formulated with sterile water or an isotonic saline or glucose solution.

The TFPI Kunitz-type domain III variant of the invention is therefore contemplated to be advantageous to use for the therapeutic applications suggested for native aprotinin or aprotinin analogues with other inhibitor profiles, in particular those which necessitate the use of large aprotinin doses. Therapeutic applications for which the use of the variant of the

[illegible] proteases, [illegible] trypsin, plasmin, kallikrein, elastase, cathepsin G and proteinase-3, include (but are not limited to) acute pancreatitis, inflammation, thrombocytopenia,

preservation of platelet function, organ preservation, wound healing, shock (including shock lung) and conditions involving hyperfibrinolytic haemorrhage, emphysema, rheumatoid arthritis, adult respiratory distress syndrome, chronic inflammatory bowel disease and psoriasis, in other words diseases presumed to be caused by pathological proteolysis by elastase, cathepsin G and proteinase-3 released from triggered PMNs.

Furthermore, the present invention relates to the use of TFPI Kunitz-type inhibitor domain III or a variant thereof as described above for the preparation of a medicament for the prevention or therapy of diseases or conditions associated with pathological proteolysis by proteases released from overstimulated PMNs. As indicated above, it may be an advantage of administer heparin concurrently with the TFPI Kunitz-type inhibitor domain III or variant.

Apart from the pharmaceutical use indicated above, TFPI Kunitz-type domain II or a variant thereof as specified above may be used to isolate useful natural substances, e.g. proteases or receptors from human material, which bind directly or indirectly to TFPI Kunitz-type domain II, for instance by screening assays or by affinity chromatography.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256
(I) TELEX: 37304

(ii) TITLE OF INVENTION: A Human Kunitz-type Protease Inhibitor Variant

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Xaa Ser Trp Cys Leu Thr Pro Ala Asp Xaa Gly Xaa Cys Xaa Xaa Xaa
1 5 10 15

Xaa Xaa Xaa Phe Tyr Tyr Asn Ser Val Ile Gly Lys Cys Arg Pro Phe
20 25 30

Xaa Tyr Xaa Gly Cys Xaa Xaa Xaa Xaa Cys

Cys Leu Arg Ala Cys Lys Lys Xaa
50 55

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly Pro Ser Trp Cys Leu Thr Pro Ala Asp Arg Gly Pro Cys Lys Ala
1 5 10 15

Arg Ile Ile Arg Phe Tyr Tyr Asn Ser Val Ile Gly Lys Cys Arg Pro
20 25 30

Phe Val Tyr Gly Gly Cys Gly Arg Lys Glu Asn Asn Phe Lys Ser Lys
35 40 45

Gln Glu Cys Leu Arg Ala Cys Lys Lys Gly
50 55

CLAIMS

1. A variant of human Kunitz-type protease inhibitor domain III of tissue factor protease inhibitor (TFPI), the variant comprising the following amino acid sequence

X^1 Ser Trp Cys Leu Thr Pro Ala Asp X^2 Gly X^3 Cys X^4 X^5 X^6 X^7 X^8 X^9 Phe Tyr Tyr Asn Ser Val Ile Gly Lys Cys Arg Pro Phe X^{10} Tyr X^{11} Gly Cys X^{12} X^{13} X^{14} Glu Asn Asn Phe X^{15} Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys Lys X^{16} (SEQ ID No. 1)

wherein X^1 represents H or 1-5 naturally occurring amino acid residues except Cys, X^2-X^{15} each independently represents a naturally occurring amino acid residue, and X^{16} represents OH or 1-5 naturally occurring amino acid residues except Cys, with the proviso that at least one of the amino acid residues X^1-X^{16} is different from the corresponding amino acid residue of the native sequence.

2. A variant according to claim 1, wherein X^1 is Gly-Pro.

3. A variant according to claim 1, wherein X^2 is an amino acid residue selected from the group consisting of Ala, Arg, Thr, Asp, Pro, Glu, Lys, Gln, Ser, Ile and Val.

4. A variant according to claim 3, wherein X^2 is Thr or Arg .

5. A variant according to claim 1, wherein X^3 is an amino acid residue selected from the group consisting of Pro, Thr, Leu, Arg, Val and Ile.

6. A variant according to claim 5, wherein X^3 is Pro or Leu

A variant according to claim 1, wherein X^4 is an amino acid residue selected from the group consisting of Lys, Arg, Val, Thr, Ile, Leu, Phe, Gly, Ser, Met, Trp, Tyr, Gln, Asn and Ala.

8. A variant according to claim 7, wherein X^4 is Lys, Val, Leu, Ile, Thr, Met, Gln or Arg.

9. A variant according to claim 1, wherein X^5 is an amino acid residue selected from the group consisting of Ala, Gly, Thr, Arg, Phe, Gln and Asp.

10. A variant according to claim 9, wherein X^5 is Ala, Thr, Asp or Gly.

11. A variant according to claim 1, wherein X^6 is an amino acid residue selected from the group consisting of Arg, Ala, Lys, Leu, Gly, His, Ser, Asp, Gln, Glu, Val, Thr, Tyr, Phe, Asn, Ile and Met.

12. A variant according to claim 11, wherein X^6 is Arg, Phe, Ala, Asn, Leu or Tyr.

13. A variant according to claim 1, wherein X^7 is an amino acid residue selected from the group consisting of Ile, Met, Gln, Glu, Thr, Leu, Val and Phe.

14. A variant according to claim 13, wherein X^7 is Ile or Glu.

15. A variant according to claim 1, wherein X^8 is an amino acid residue selected from the group consisting of Ile, Thr, Leu, Asn, Lys, Ser, Gln, Glu, Arg, Pro and Phe.

16. A variant according to claim 15, wherein X^8 is Ile or Asn.

17. A variant according to claim 1, wherein X^9 is an amino acid residue selected from the group consisting of Arg, Ser, Ala, Gln, Lys and Leu.

18. A variant according to claim 17, wherein X^9 is Arg.

19. A variant according to claim 1, wherein X^{10} is an amino acid

residue selected from the group consisting of Gln, Pro, Phe, Ile Lys, Trp, Ala, Thr, Leu, Ser, Tyr, His, Asp, Met, Arg and Val.

20. A variant according to claim 19, wherein X^{10} is Val or Lys.

21. A variant according to claim 1, wherein X^{11} is Ser or Gly.

22. A variant according to claim 1, wherein X^{12} is an amino acid residue selected from the group consisting of Gly, Met, Gln, Glu, Leu, Arg, Lys, Pro and Asn.

23. A variant according to claim 22, wherein X^{12} is Ala or Leu.

24. A variant according to claim 1, wherein X^{13} is Ala or Gly.

25. A variant according to claim 1, wherein X^{14} is an amino acid residue selected from the group consisting of Lys, Asn and Asp.

26. A variant according to claim 25, wherein X^{14} is Lys or Asn.

27. A variant according to claim 1, wherein X^{15} is an amino acid residue selected from the group consisting of Val, Tyr, Asp, Glu, Thr, Gly, Leu, Ser, Ile, Gln, His, Asn, Pro, Phe, Met, Ala, Arg, Trp and Lys.

28. A variant according to claim 27, wherein X^{15} is Lys or Glu.

29. A variant according to claim 1, wherein X^{16} is Gly.

30. A variant according to claim 1, wherein X^{1} is Gly-Pro and X^{16} is Gly.

31. A variant according to claim 1 comprising the following

Gly Pro Ser Trp Cys Leu Thr Pro Ala Asp Arg Gly Pro Cys Lys Ala
Arg Ile Ile Arg Phe Tyr Tyr Asn Ser Val Ile Gly Lys Cys Arg Pro

Phe Val Tyr Gly Gly Cys Gly Arg Lys Glu Asn Asn Phe Lys Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys Lys Gly (SEQ ID No. 2).

32. A DNA construct comprising a DNA sequence encoding a human Kunitz-type protease inhibitor variant according to any of claims 1-31.

33. A recombinant expression vector comprising a DNA construct according to claim 32.

34. A cell containing a DNA construct according to claim 32 or an expression vector according to claim 33.

35. A method of producing a human Kunitz-type protease inhibitor variant according to any of claims 1-31, the method comprising culturing a cell according to claim 34 under conditions conducive to the expression of the protein, and recovering the resulting protein from the culture.

36. A pharmaceutical composition comprising a human Kunitz-type protease inhibitor variant according to any of claims 1-31 and a pharmaceutically acceptable carrier or excipient.

37. A composition according to claim 36 which further comprises heparin.

38. Use of human Kunitz-type protease inhibitor domain III of TFPI or a variant thereof according to any of claims 1-31 for the preparation of a medicament for the prevention or treatment of diseases or conditions associated with pathological proteolysis.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00003

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C07K 7/10, C12N 15/15, A61K 37/64
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CHEMICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, Volume 338, April 1989, Thomas J. Girard et al, "Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor"	1,7,8,35,36, 38
A	--	2-6,9-34
A,P	US, A, 5106833 (BROZE, JR. ET AL), 21 April 1992 (21.04.92)	1-38
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☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

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7 April 1993

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Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer
Elisabeth Carlborg
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

Information on patent family members

26/02/93

International application No.
PCT/DK 93/00003

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 5106833	21/04/92	AU-B- 604138	06/12/90
		AU-A- 1928688	02/02/89
		EP-A- 0300988	25/01/89
		AU-A- 1928788	25/05/89
		EP-A- 0318451	31/05/89